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RNA Purification

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Selecting a Purification Strategy	198
Do Your Experiments Require Total RNA or mRNA?	198
Is It Possible to Predict the Total RNA Yield from a Certain Mass of Tissue or Number of Cells?	201
Is There Protein in Your RNA Preparation, and If So, Should You Be Concerned?	202
Is Your RNA Physically Intact? Does It Matter?	202
Which Total RNA Isolation Technique Is Most Appropriate for Your Research?	203
What Protocol Modifications Should Be Used for RNA Isolation from Difficult Tissues?	207
Is a One-Step or Two-Step mRNA-(poly(A) RNA)- Purification Strategy Most Appropriate for Your Situation?	209
How Many Rounds of Oligo(dT)–Cellulose Purification Are Required?	210
Which Oligo(dT)–Cellulose Format Is Most Appropriate?	210
Can Oligo(dT)–Cellulose Be Regenerated and Reused? ...	211
Can a Kit Designed to Isolate mRNA Directly from the Biological Sample Purify mRNA from Total RNA? ...	212
Maximizing the Yield and Quality of an RNA Preparation ...	212
What Constitutes “RNase-Free Technique”?	212

How Does DEPC Inhibit RNase?	213
How Are DEPC-Treated Solutions Prepared? Is More DEPC Better?	213
Should You Prepare Reagents with DEPC-Treated Water, or Should You Treat Your Pre-made Reagents with DEPC?	214
How Do You Minimize RNA Degradation during Sample Collection and Storage?	214
How Do You Minimize RNA Degradation during Sample Disruption?	215
Is There a Safe Place to Pause during an RNA Purification Procedure?	218
What Are the Options to Quantitate Dilute RNA Solutions?	218
What Are the Options for Storage of Purified RNA?	219
Troubleshooting	220
A Pellet of Precipitation RNA Is Not Seen at the End of the RNA Purification	220
A Pellet Was Generated, but the Spectrophotometer Reported a Lower Reading Than Expected, or Zero Absorbance	221
RNA Was Prepared in Large Quantity, but it Failed in a Downstream Reaction: RT PCR is an Example	221
My Total RNA Appeared as a Smear in an Ethidium Bromide-stained Denaturing Agarose Gel; 18S and 28S RNA Bands Were not Observed	222
Only a Fraction of the Original RNA Stored at -70°C Remained after Storage for Six Months	222
Bibliography	222

SELECTING A PURIFICATION STRATEGY

Do Your Experiments Require Total RNA or mRNA?

One of the first decisions that the researcher has to make when detecting or quantitating RNA is whether to isolate total RNA or poly(A)-selected RNA (also commonly referred to as mRNA). This choice is further complicated by the bewildering array of RNA isolation kits available in the marketplace. In addition the downstream application influences this choice. The following section is a short primer in helping make that decision.

From a purely application point of view, total RNA might suffice for most applications, and it is frequently the starting material for applications ranging from the detection of an mRNA species by Northern hybridization to quantitation of a message by

RT-PCR. The preference for total RNA reflects the challenge of purifying enough poly(A) RNA for the application (mRNA comprises <5% of cellular RNA), the potential loss of a particular message species during poly(A) purification, and the difficulty in quantitating small amounts of purified poly(A) RNA. If the data generated with total RNA do not meet your expectations, using poly(A) RNA instead might provide the sensitivity and specificity that your application requires. The pros and cons with either choice are discussed below. Your experimental data will provide the best guidance in deciding whether to use total or poly(A) RNA. Be flexible and open minded; there are many variables to consider when making this decision.

Two situations where using poly(A) RNA is essential are cDNA library construction, and preparation of labeled cDNA for hybridization to gene arrays. To avoid generating cDNA libraries with large numbers of ribosomal clones, and nonspecific labeled cDNA it is crucial to start with poly(A) RNA for these procedures.

The next section gives a brief description of the merits and demerits of using total RNA or poly(A) RNA in some of the most common RNA analysis techniques. Chapter 14, "Nucleic Acid Hybridization," discusses the nuances and quirks of these procedures in greater depth. For detailed RNA purification protocols, see Krieg (1996) Rapley and Manning (1998), and Farrel (1998).

Northern Hybridizations

Northern analysis is the only technique available that can determine the molecular weight of an mRNA species. It is also the least sensitive. Total RNA is most commonly used in this assay, but if you don't detect the desired signal, or if false positive signals from ribosomal RNA are a problem, switching to poly(A) RNA might be a good idea. Since only very small amounts of poly(A) RNA are present, make sure that it is feasible and practical to obtain enough starting cells or tissue. Theoretically you could use as much as 30 μ g of poly(A) RNA in a Northern, which is the amount found in approximately 1 mg of total RNA. Will it be practical and feasible for you to sacrifice the cells or tissue required to get this much RNA? If not, use as much poly(A) RNA as is practical.

One drawback to using poly(A) RNA in Northern hybridizations is the absence of the ribosomal RNA bands, which are ordinarily used to gauge the quality and relative quantity of the RNA samples, as discussed later in this chapter. Fortunately there are other strategies besides switching to poly(A) RNA that can be used to increase the sensitivity of Northern hybridizations. You could alter the hybridization conditions of the DNA probe

(Anderson, 1999), or you could switch to using RNA probes in the hybridization, which are 3- to 5-fold more sensitive than DNA probes in typical hybridization buffers (Ambion Technical Bulletin 168, and references therein). Dramatic differences in the sensitivity of Northern blots can also be seen from using different hybridization buffers.

If you remain dissatisfied with the Northern data, and you are not interested in determining the size of the target, switching to a more sensitive technique such as nuclease protection or RT-PCR might help. Nuclease protection assays, which are 5- to 10-fold more sensitive than traditional membrane hybridizations, can accommodate 80 to 100 μg of nucleic acid in a single experiment. RT-PCR can detect extremely rare messages, for example, 400 copies of a message in a 1 μg sample as described by Sun et al. (1998). RT-PCR is currently the most sensitive of the RNA analysis techniques, enabling detection and quantitation of the rarest of targets. Quantitative approaches have become increasingly reliable with introduction of internal standards such as in competitive PCR strategies (Totzke et al., 1996; Riedy et al., 1995).

Dot/Slot Blots

In this procedure, RNA samples are directly applied to a membrane, either manually or under vacuum through a filtration manifold. Hybridization of probe to serial dilutions of sample can quickly generate quantitative data about the expression level of a target. Total RNA or poly(A) RNA can be used in this assay. Since the RNA is not size-fractionated on an agarose gel, a potential drawback to using total RNA in dot/slot blots is that signal of interest cannot be distinguished from cross-hybridization to rRNA. Switching to poly(A) RNA as the target source might alleviate this problem. However, it is crucial that relevant positive and negative controls are run with every dot/slot blot, whether the source of target nucleic acid is total RNA or poly(A) RNA.

Hybridization to Gene Arrays and Reverse Dot Blots

Gene arrays consist of cDNA clones (sometimes in the form of PCR products, sometimes as oligonucleotides) or the corresponding oligos spotted at high density on a nylon membrane, glass slide, or other solid support. By hybridizing labeled cDNA probes reverse transcribed from mRNA, the expression of potentially hundreds of genes can be simultaneously analyzed. This procedure requires that the labeled cDNA be present in excess of the target spotted on the array. This is difficult to achieve unless poly(A) RNA is used as template in the labeling reaction.

Ribonuclease Protection Assays

Either total RNA or poly(A) RNA can be used as starting material in nuclease protection assays. However, total RNA usually affords enough sensitivity to detect even rare messages, when the maximum amount (as much as 80 to 100 μg) is used in the assay. If the gene is expressed at extremely low levels, requiring week-long exposure times for detection, a switch to poly(A) RNA might prove beneficial and may justify the added cost. Although very sensitive, nuclease protection assays do require laborious gel purification of the full-length probe to avoid getting confusing results.

RT-PCR

RT-PCR is the most sensitive method for detecting and quantitating mRNA. Theoretically, even very low-abundance messages can be detected with this technique. Total RNA is routinely used as the template for RT-PCR, (Frohman, 1990) but some cloning situations and rare messages require the use of poly(A) RNA (Amersham Pharmacia Biotech, 1995).

Note that one school of thought concerning RT-PCR considers it advisable to treat the sample RNA with DNase I, since no purification method produces RNA completely free of contaminating genomic DNA. RT-PCR is sensitive enough that even very small amounts of genomic DNA contamination can cause false positives. A second school of thought preaches avoidance of DNase I, as discussed in Chapter 11, "PCR."

cDNA Library Synthesis

As mentioned earlier, high-quality mRNA that is essentially free of ribosomal RNA is required for constructing cDNA libraries. Unacceptably high backgrounds of ribosomal RNA clones would be produced if total RNA were reverse transcribed to prepare cDNA.

Is It Possible to Predict the Total RNA Yield from a Certain Mass of Tissue or Number of Cells?

The data provided in this section are based on experimentation at Ambion, Inc. using a variety of samples and different purification products. The reader is cautioned that these are theoretical estimates, and yields can vary widely based on the type of tissue or cells used for the isolation, especially when dealing with difficult samples, as discussed later. The importance of rapid and complete tissue disruption, and homogenizing at subfreezing tem-

peratures cannot be overemphasized. In addition, yields from very small amounts of starting material are subject to the law of diminishing returns. Thus, if the option is available, always choose more starting material rather than less. Samples can be pooled together, if possible, to maximize yields.

For example, 5 mg of tissue or 2.5×10^6 cells yields about 10 μg of total RNA, comprised of 8 μg rRNA, 0.3 μg mRNA, 1.7 μg tRNA, and other RNA. In comparison, 1 g of tissue or 5×10^8 cells yields about 2 mg of total RNA, comprised of 1.6 mg rRNA + 60 μg mRNA + 333 μg tRNA and other RNA.

Is There Protein in Your RNA Preparation, and If So, Should You Be Concerned?

Pure RNA has an $A_{260}:A_{280}$ absorbance ratio of 2.0. However, for most applications, a low $A_{260}:A_{280}$ ratio probably won't affect the results. Researchers at Ambion, Inc. have used total RNA with $A_{260:280}$ ratios ranging from 1.4 to 1.8 with good results in RNase protection assays, Northern analysis, in vitro translation experiments, and RT-PCR assays. If protein contamination is suspected to be causing problems, additional organic extractions with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 mixture) may remove the contaminant. Residual phenol can also lower the $A_{260}:A_{280}$ ratio, and inhibit downstream enzymatic reactions. Chloroform/isoamyl alcohol (24:1) extraction will remove residual phenol. Chapter 4, "How to Properly Use And Maintain Laboratory Equipment," discusses other artifacts that raise and lower the $A_{260:280}$ ratio. Some tissues will consistently produce RNA with a lower $A_{260:280}$ ratio than others; the $A_{260:280}$ ratio for RNA isolated from liver and kidney tissue, for example, is rarely above 1.7.

Is Your RNA Physically Intact? Does It Matter?

The integrity of your RNA is best determined by electrophoresis on a formaldehyde agarose gel under denaturing conditions. The samples can be visualized by adding 10 $\mu\text{g}/\text{ml}$ of Ethidium Bromide (EtBr) (final concentration) to the sample before loading on the gel. Compare your prep's 28S rRNA band (located at approximately 5 Kb in most mammalian cells) to the 18S rRNA band (located at approximately 2.0 Kb in most mammalian cells). In high-quality RNA the 28S band should be approximately twice the intensity of the 18S band (Figure 8.1).

The most sensitive test of RNA integrity is Northern analysis using a high molecular weight probe expressed at low levels in the tissues being analyzed. However, this method of quality control is very time-consuming and is not necessary in most cases.

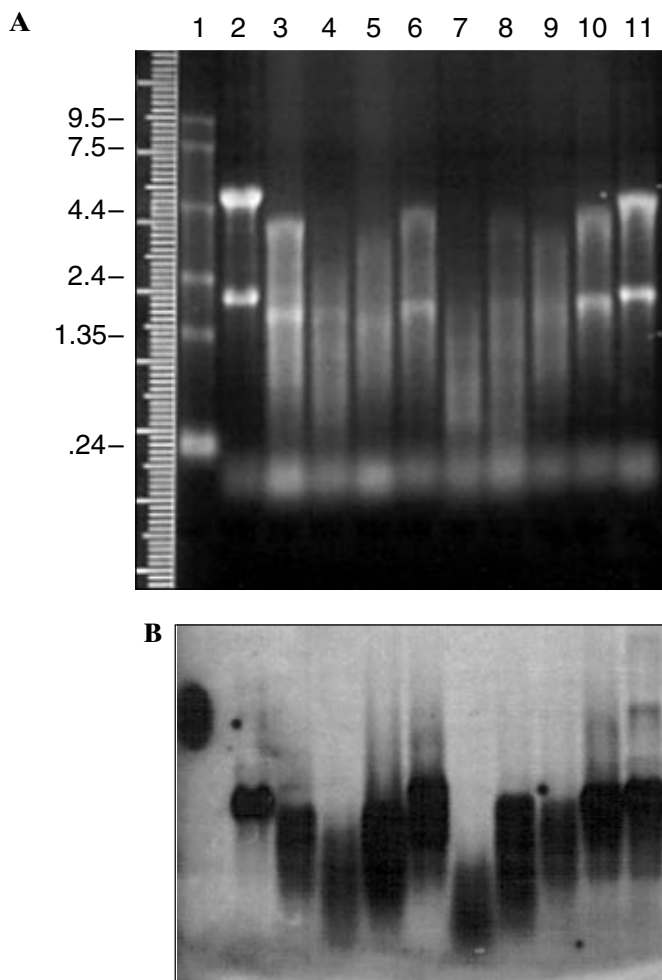


Figure 8.1 Assessing quality of RNA preparation via agarose gel electrophoresis (A) This gel shows total RNA samples (5 µg/lane) ranging from high-quality, intact RNA (lane 2) to almost totally degraded RNA (lane 7). Note that as the RNA is degraded, the 28S and 18S ribosomal bands become less distinct, the intensity of the ribosomal bands relative to the background staining in the lane is reduced, and there is a significant shift in their apparent size as compared to the size standards. (B) This is an autoradiograph of the same gel after hybridization with a biotinylated GAPDH RNA probe followed by nonisotopic detection. The exposure is 10 minutes the day after the chemiluminescent substrate was applied. Note that the signal in lane 2, from intact RNA, is well localized with minimal smearing, whereas the signals from degraded RNA samples show progressively more smearing below the bands, or when the RNA is extremely degraded, no bands at all (lane 7). Reprinted by permission of Ambion, Inc.

Northern analysis is not tolerant of partially degraded RNA. If samples are even slightly degraded, the quality of the data is severely compromised. For example, even a single cleavage in 20% of the target molecules will decrease the signal on a Northern blot by 20%. Nuclease protection assays and RT-PCR analyses will tolerate partially degraded RNA without compromising the quantitative nature of the results.

Which Total RNA Isolation Technique Is Most Appropriate for Your Research?

There are three basic methods of isolating total RNA from cells and tissue samples. Most rely on a chaotropic agent such as guanidium or a detergent to break open the cells and simultaneously

inactivate RNases. The lysate is then processed in one of several ways to purify the RNA away from protein, genomic DNA, and other cellular components. A brief description of each method along with the time and effort involved, the quality of RNA obtained, and the scalability of the procedures follow.

Guanidium-Cesium Chloride Method

Slow, laborious procedure, but RNA is squeaky clean; unsuitable for large sample numbers; little if any genomic DNA remains.

This method employs guanidium isothiocyanate to lyse cells and simultaneously inactivate ribonucleases rapidly. The cellular RNA is purified from the lysate via ultracentrifugation through a cesium chloride or cesium trifluoroacetate cushion. Since RNA is more dense than DNA and most proteins, it pellets at the bottom of the tube after 12 to 24 hours of centrifugation at $\geq 32,000$ rpm.

This classic method yields the highest-quality RNA of any available technique. Small RNAs (e.g., 5S RNA and tRNAs) cannot be prepared by this method as they will not be recovered (Mehra, 1996). The original procedures were time-consuming, laborious, and required overnight centrifugation. The number and size of samples that could be processed simultaneously were limited by the number of spaces in the rotor. Commercial products have been developed to replace this lengthy centrifugation (Paladichuk, 1999) with easier, less time-consuming methods. However, if the goal were to isolate very high-quality RNA from a limited number of samples, this would be the method of choice (Glisin, Crkuenjakov and Byus, 1974).

Single- and Multiple Step Guanidium Acid-Phenol Method

Faster, fewer steps, prone to genomic DNA contamination, somewhat cumbersome if large sample numbers are to be processed.

The guanidium-acid phenol procedure has largely replaced the cesium cushion method because RNA can be isolated from a large number of samples in two to four hours (although somewhat cumbersome) without resorting to ultracentrifugation. RNA molecules of all sizes are purified, and the technique can be easily scaled up or down to process different sample sizes. The single-step method (Chomczynski and Sacchi, 1987) is based on the propensity of RNA molecules to remain dissolved in the aqueous phase in a solution containing 4M guanidium thiocyanate, pH 4.0, in the presence of a phenol/chloroform organic phase. At this low pH, DNA molecules remain in the organic phase, whereas proteins and other cellular macromolecules are retained at the interphase.

It is not difficult to find researchers who swear by GITC—phenol procedures because good-quality RNA, free from genomic DNA contamination is quickly produced. However, a second camp of researchers avoid these same procedures because they often contain contaminating genomic DNA (Lewis, 1997; S. Herzer, personal communication). There is no single explanation for these polarized opinions, but the following should be considered.

Problems can occur in the procedure during the phenol/chloroform extraction step. The mixture must be spun with sufficient force to ensure adequate separation of the organic and aqueous layers; this will depend on the rotor as can be seen in Table 8.1. For best results the centrifuge brake should not be applied, nor should it be applied to gentler settings.

The interface between the aqueous and organic layers is another potential source of genomic contamination. To get high-purity RNA, avoid the white interface (can also appear cream colored or brownish) between the two layers; leave some of the aqueous layer with the organic layer. If RNA yield is crucial, you'll probably want as much of the aqueous layer as possible, again leaving the white interface. In either case you can repeat the organic extraction until no white interface is seen.

Residual salt from the precipitation step, appearing as a huge white pellet, can interfere with subsequent reactions. Excessive salt should be suspected when a very large white pellet is obtained from an RNA precipitation. Excess salt can be removed by washing the RNA pellet with 70% EtOH (ACS grade). To the RNA pellet, add about 0.3ml of room temperature (or -20°C) 70% ethanol per 1.5ml tube or approximately 2 to 3ml per 15 to 40ml tube. Vortex the tube for 30 seconds to several minutes to dislodge the pellet and wash it thoroughly. Recover the RNA with a low speed spin, (approximately $3000 \times g$; approximately 7500rpm in a microcentrifuge, or approximately 5500rpm in a SS34 rotor), for 5 to 10 minutes at room temperature or at 4°C .

Table 8.1 Spin Requirements for Phenol Chloroform Extractions

Volume Tube	Speed	Spin Time
1.5ml	$10,000 \times g$	5 minutes
2.0ml	$12,000 \times g$	5 minutes
15ml	$12,000 \times g$	15 minutes
50ml	$12,000 \times g$	15 minutes

Remove the ethanol carefully, as the pellets may not adhere tightly to the tubes. The tubes should then be respun briefly and the residual ethanol removed by aspiration with a drawn out Pasteur pipet. Repeat this wash if the pellet seems unusually large.

Non-Phenol-Based Methods

Very fast, clean RNA, can process large sample numbers, possible genomic contamination.

One major drawback to using the guanidium acid-phenol method is the handling and disposal of phenol, a very hazardous chemical. As a result phenol-free methods, based on the ability of glass fiber filters to bind nucleic acids in the presence of chaotropic salts like guanidium, have gained favor. As with the other methods, the cells are first lysed in a guanidium-based buffer. The lysate is then diluted with an organic solvent such as ethanol or isopropanol and applied to a glass fiber filter or resin. DNA and proteins are washed off, and the RNA is eluted at the end in an aqueous buffer.

This technique yields total RNA of the same quality as the phenol-based methods. DNA contamination can be higher with this method than with phenol-based methods (Ambion, Inc., unpublished observations). Since these are column-based protocols requiring no organic extractions, processing large sample numbers is fast and easy. This is also among the quickest methods for RNA isolation, usually completed in less than one hour.

The primary problem associated with this procedure is clogging of the glass fiber filter by thick lysates. This can be prevented by using a larger volume of lysis buffer initially. A second approach is to minimize the viscosity of the lysate by sonication (on ice, avoid power settings that generate frothing) or by drawing the lysate through an 18 gauge needle approximately 5 to 10 times. This step is more likely to be required for cells grown in culture than for lysates made from solid tissue. If you are working with a tissue that is known to be problematic (i.e., high in saccharides or fatty acids), an initial clarifying spin or extraction with an equal volume of chloroform can prevent filter-clogging problems. A reasonable starting condition for the clarifying spin is 8 minutes at $7650 \times g$. If a large centrifuge is not available, the lysate can be divided into microcentrifuge tubes and centrifuged at maximum speed for 5 to 10 minutes. Avoid initial clarifying spins on tissues rich in glycogen such as liver, or plants containing high molecular-weight carbohydrates. If you generate a clogged filter, remove the remainder of the lysate using a pipettor, place it on top of a fresh filter, and continue with the isolation protocol using both filters.

What Protocol Modifications Should Be Used for RNA Isolation from Difficult Tissues?

RNA isolation from some tissues requires protocol modifications to eliminate specific contaminants, or tissue treatments prior to the RNA isolation protocol. Fibrous tissues and tissue rich in protein, DNA and RNases, present unique challenges for total RNA isolation. In this section we address problems presented by difficult tissues and offer troubleshooting techniques to help overcome these problems. A separate section will discuss the homogenization needs of various sample types in greater detail.

Web sites that discuss similar issues are <http://www.nwfsc.noaa.gov/protocols/methods/RNAMethodsMenu.html> and <http://grimwade.biochem.unimelb.edu.au/sigtrans.html>.

Fibrous Tissue

Good yields and quality of total RNA from fibrous tissue such as heart and muscle are dependent on the complete disruption of the starting material when preparing homogenates. Due to low cell density and the polynucleate nature of muscle tissue, yields are typically low; hence it is critical to make the most of the tissue at hand. Pulverizing the frozen tissue into a powder while keeping the tissue completely frozen (use a chilled mortar and pestle) is the key to isolating intact total RNA. It is critical that there be no discernible lumps of tissue remaining after homogenization.

Lipid and Polysaccharide-Rich Tissue

Plant and brain tissues are typically rich in lipids, which makes it difficult to get clean separation of the RNA and the rest of the cellular debris. When using phenol-based methods to isolate total RNA, white flocculent material present throughout the aqueous phase is a classic indicator of this problem. This flocculate will not accumulate at the interface even after extended centrifugation. Chloroform:isoamyl alcohol (24:1) extraction of the lysate is probably the best way to partition the lipids away from the RNA. To minimize loss, back-extract the organic phase, and then clean up the recovered aqueous RNA by extraction with phenol:chloroform:isoamyl alcohol (25:24:1).

When isolating total RNA from plant tissue using a non-phenol-based method, polyvinylpyrrolidone-40 (PVP-40) can be added to the lysate to absorb polysaccharide and polyphenolic contaminants. When the lysate is centrifuged to remove cell debris, these contaminants will be pelleted with the PVP (Fang, Hammar, and Grumet, 1992; see also the chapter by Wilkins and Smart, "Isolation of RNA from Plant Tissue," in Krieg, 1996, for a list of refer-

ences and protocols for removing these contaminants from plant RNA preps). Centrifugation on cesium trifluoroacetate has also been shown to separate carbohydrate complexes from RNA (Zarlenga and Gamble, 1987).

Nucleic Acid and Nuclease-Rich Tissue

Spleen and thymus are high in both nucleic acids and ribonuclease. Good homogenization is the key to isolating high-quality RNA from these tissues. Tissue samples should be completely pulverized on dry ice, under liquid nitrogen, to facilitate rapid homogenization in the lysis solution, which inhibits nucleases. Cancerous cells and cell lines also contain high amounts of DNA and RNA, which makes them unusually viscous, causing poor separation of the organic and aqueous phases and potentially clogging RNA-binding filters. Increasing the ratio of sample mass: volume of lysis buffer can help alleviate this problem in filter-based isolations. Multiple acid-phenol extractions can be done to ensure that most of the DNA is partitioned into the organic phase during acid-phenol-based isolation procedures. Two to three extractions are usually sufficient; one can easily tell if a lysate is viscous by attempting to pipet the solution and observing whether it sticks to the pipette tip. The DNA in the lysate can alternatively be sheared, either by vigorous and repeated aspiration through a small gauge needle (18 gauge) or by sonication (10 second sonication at 1/3 maximum power on ice, or until the viscosity is reduced).

Hard Tissue

Hard tissue, such as bone and tree bark, cannot be effectively disrupted using a PolytronTM or any other commonly available homogenizer. In this case heavy-duty tissue grinders that pulverize the material using mechanical force are needed. SPEX Certiprep, Metuchen, NJ, makes tissue-grinding mills that chill samples to liquid nitrogen temperatures and pulverize them by shuttling a steel piston back and forth inside a stationary grinding vial.

Bacteria and Yeast

Bacterial and yeast cells can prove quite refractory to isolating good-quality RNA due to the difficulty of lysing them. Another problem with bacteria is the short half-life of most bacterial messages. Lysis can be facilitated by resuspending cell pellets in TE and treating with lysozyme, subsequent to which the actual

extraction steps are performed. A potential drawback of using lytic enzymes is that they can introduce RNases. Use the highest-quality enzymes to reduce the likelihood of introducing contaminants. Yield and quality from phenol-based extraction protocols can also be improved by conducting the organic extractions at high temperatures (Lin et al., 1996).

Lysis of yeast cells is accomplished by vigorous vortexing in the presence of 0.4 to 0.5 mm glass beads. If using a non-phenol-based procedure for RNA isolation, the lysis can be monitored by looking for an increase in A_{260} readings. Yeast cells can also be treated with enzymes such as zymolase, lyticase, and glucolase to facilitate lysis (Ausubel et al., 1995).

Is a One-Step or Two-Step mRNA–(poly(A) RNA)–Purification Strategy Most Appropriate for Your Situation?

One-step procedures purify poly(A) RNA directly from the starting material. A two-step strategy first isolates total RNA, and then purifies poly(A) RNA from that.

Sample Number

One-step strategies involve fewer manipulations to recover poly(A) RNA. When comparing different one-step strategies, consider that two additional washing steps multiplied by 20 samples can consume significant time and materials, and arguably, faster purification strategies decrease the chance of degradation. Centrifugation, magnetics, and other technologies sound appealing and fast, but the true speed of a technique is determined by the total manipulations in a procedure. High-throughput applications such as hybridization of gene arrays are usually best supported by one-step purification procedures.

Sample Mass

The percentage of poly(A) RNA recovery is similar between one- and two-step strategies. So, when experimental sample is limited, a one-step procedure is usually the more practical procedure.

Yield

Commercial one-step products are usually geared to purify small (1–5 μg) or large (25 μg) quantities of poly(A) RNA, and manufacturers can usually provide data generated from a variety of sample types. If you require more poly(A) RNA, a two-step procedure is usually more cost effective.

How Many Rounds of Oligo(dT)–Cellulose Purification Are Required?

One round of poly(A) RNA selection via oligo(dT)–cellulose typically removes 50 to 70% of the ribosomal RNA. One round of selection is adequate for most applications (i.e., Northern analysis and ribonuclease protection assays). A cDNA library generated from poly(A) RNA that is 50% pure is usually sufficient to identify most genes, but to generate cDNA libraries with minimal rRNA clones, two rounds of oligo(dT) selection will remove approximately 95% of the ribosomal RNA. Remember that 20 to 50% of the poly(A) RNA can be lost during each round of oligo(dT) selection, so multiple rounds of selection will decrease your mRNA yield. The use of labeled cDNA to screen gene arrays is severely compromised by the presence of rRNA-specific probes, so two rounds of poly(A) selection might be justified.

Which Oligo(dT)–Cellulose Format Is Most Appropriate?

Resins

Commercial resins are derivatized with oligo(dT) of various lengths at various loading capacities—mass of oligo(dT) per mass of cellulose. The linkage between the oligo(dT) and cellulose is strong but not covalent; some nucleic acid will leave the resin during use. Oligo(dT) chains 20 to 50 nucleotides long, bound to cellulose at loading capacities of approximately 50 mg/ml, are commonly used in column and batch procedures. Some suppliers refer to this as Type 7 oligo(dT)-cellulose. The word “Type” refers to the nature of the cellulose. Type 77F cellulose is comprised of shorter strands than Type 7, and it does not provide good flow in a chromatography column. Type 77F does work very well in a batch mode, binding more mRNA than Type 7.

Column Chromatography

Oligo(dT)-cellulose can be scaled up or down using a variety of column sizes. Column dimension isn't crucial, but the frit or membrane that supports the oligo(dT)-cellulose is. The microscopic cellulose fibers can clog the frits and filter discs in a gravity chromatography column. Test the ability of several ml of buffer or water to flow through your column before adding your RNA sample. If your column becomes clogged during use, resuspend the packed resin with gentle mixing, and prepare a new column using a different frit, or do a batch purification on the rescued resin as described below. Some commercial products pack oligo(dT)-cellulose in a syringelike system so that the plunger can forcefully

push through the matrix. The frits in these push-systems accommodate flow under pressure. Applying pressure to a clogged, standard oligo(dT)-cellulose chromatography column usually worsens matters. Occasionally air bubbles become trapped within the spaces of the frit. Gentle pressure or a very gentle vacuum applied to the exit port of the column can release these trapped bubbles and improve flow.

Batch Binding or Spin Columns

Batch binding consists of directly mixing the total RNA with oligo(dT)-cellulose in a centrifuge tube, and using a centrifuge to separate the cellulose from the supernate in the wash and elution steps. Batch binding and washing of the matrix and spun columns circumvent the problems of slow flow rates, and clogged columns often experienced with gravity-driven chromatography. Scaling reactions up and down is convenient and economical, using the guidelines of 100 A₂₆₀ units of total RNA per 0.5 g of oligo(dT)-cellulose. Increasing the incubation times for the poly(A) RNA hybridization to the oligo(dT)-cellulose can sometimes increase yields by 5 to 10%.

Tissues that lyse only with difficulty, and viscous lysates, can interfere with oligo(dT) binding by impeding the movement of oligo(dT)-coated particles. Additional lysis buffer, or repeated passage through a fine-gauge (21 gauge) needle with a syringe to shear the DNA and proteins, can reduce this viscosity. Lysates with excessive amounts of particulates should be cleared by centrifugation before attempting to select poly(A) RNA.

Can Oligo(dT)-Cellulose Be Regenerated and Reused?

Oligo(dT)-cellulose can theoretically be regenerated and reused, but the reader is strongly recommended not to do so. The hydroxide wash that regenerates the resin should destroy any lingering mRNA, but it is difficult to prove 100% destruction. Also the more a reagent is manipulated, the more likely it is to become contaminated with trace amounts of RNase. However some researchers still reuse oligo(dT)-cellulose until poor flow or reduced binding leads them to prepare fresh oligo(dT)-cellulose. Be especially wary of regenerated oligo(dT)-cellulose that appears pink or slimy.

If you must reuse oligo(dT)-cellulose, first wash it with 10 bed volumes of elution buffer followed by 10 bed volumes of 0.1N NaOH. (One bed volume equals the volume of cellulose settled in the column.) The NaOH degrades any RNA remaining after elution. After the 0.1N NaOH treatment, wash the oligo(dT)-

cellulose with 10 bed volumes of water followed by 10 bed volumes of absolute alcohol. If the regenerated oligo(dT)-cellulose is to be stored for longer than a couple of weeks, dry it under a vacuum and store it with desiccant at -20°C . For short-term storage, refrigerate at 4°C after the NaOH and water washes; desiccation isn't required.

If the oligo(dT)-cellulose is to be reused immediately after removing residual RNA with the NaOH wash, equilibrate the column in 10 bed volumes of elution buffer followed by 10 bed volumes of binding buffer. The column is now ready for sample application.

To use resin that has previously been regenerated and stored, resuspend the oligo(dT)-cellulose in elution buffer, pour into the column, and wash with 10 bed volumes of binding buffer.

Can a Kit Designed to Isolate mRNA Directly from the Biological Sample Purify mRNA from Total RNA?

One-step procedures that obtain mRNA from intact cells or tissue typically employ a denaturing solution to generate a lysate, which is directly added to the oligo(dT)-cellulose. Washing with specific concentrations of salt buffers ultimately separates poly(A) RNA from DNA and other RNA species.

Typically total RNA can be substituted into one-step procedures by skipping the homogenization steps, adjusting the salt concentration of the total RNA to 500 mM and adding this material to the oligo(dT)-cellulose. Consult the manufacturer of your product for their opinion on this approach, and verify the binding capacity of the oligo(dT)-cellulose for total RNA.

MAXIMIZING THE YIELD AND QUALITY OF AN RNA PREPARATION

What Constitutes "RNase-Free Technique"?

Fundamentals

RNase contamination is so prevalent, special attention must be given to the preparation of solutions. Solutions should be prepared in disposable, RNase-free plasticware or in RNase-free glassware prepared in the lab. Glassware can be made RNase-free by baking at 180°C for 8 hours to overnight, or by treating with a commercial RNase decontaminating solution. Alternatively, RNase can be removed by filling containers with 0.1% DEPC, incubating at 37°C for 2 hours, rinsing with sterile water and

then either heating to 100°C for 15 minutes, or autoclaving for 15 minutes to eliminate RNase.

Electrophoresis apparatus used for RNA analysis can be made RNase-free by filling with a 3% hydrogen peroxide solution, incubating for 10 minutes at room temperature and rinsing with DEPC-treated water.

When preparing RNase-free solutions, wear gloves and change them often. Regardless of the method used to prepare RNase-free solutions, keep in mind that they can easily become contaminated after preparation. This occurs when solutions are open and used regularly, or when they are shared with others. It is wise to prepare small volumes of solutions and aliquot larger volumes into RNase-free containers. Solutions should be clearly labeled “RNase-free” to avoid contamination and should only be used with RNase-free pipettes and pipette tips. Also adhere to the maxim “when in doubt, throw it out.”

How Does DEPC Inhibit RNase?

The most common method of preparing RNase-free solutions is diethylpyrocarbonate (DEPC) treatment. DEPC inactivates RNases by carboxyethylation of specific amino acid side chains in the protein (Brown, 1991). DEPC is a suspected carcinogen, and it should always be used with the proper precautions.

How Are DEPC-Treated Solutions Prepared? Is More DEPC Better?

Most protocols specify adding DEPC to solutions at a concentration 0.1%, followed by mixing and room temperature incubation for several hours to overnight. The container lid should be loosened for the extended incubation because a considerable amount of pressure can form during the reaction. Finally, the solution is autoclaved; this inactivates the residual DEPC by hydrolysis, and releases CO₂ and EtOH as by-products.

The EtOH by-product can combine with trace carboxylic acid contaminants in the vessel to form volatile esters, which impart a slightly fruity smell to the solution. This does not mean that trace DEPC remains in solution. DEPC has a half-life of 30 minutes in water, and at a DEPC concentration of 0.1%, solutions autoclaved for 15 minutes/liter can be assumed to be DEPC-free. Be aware that increasing the concentration of DEPC to 1% can inhibit more RNase but can also inhibit certain enzymatic reactions, so more is usually not better.

Should You Prepare Reagents with DEPC-Treated Water, or Should You Treat Your Pre-made Reagents with DEPC?

Some researchers prefer to DEPC-treat preprepared solutions, while others opt for preparing DEPC-treated water first and combining it with ultrapure RNase-free powdered reagents. It should be noted that many reagents commonly used in RNA studies contain primary amines, such as Tris, MOPS, HEPES, and PBS, and cannot be DEPC-treated because the amino group “sops up” the DEPC, making it unavailable to inactivate RNase. These solutions should be prepared with ultrapure reagents and DEPC-treated water. When preparing solutions in this manner, use RNase-free spatulas and magnetic stirrers, wear gloves and change them often. Spatulas and magnetic stirrers can be made RNase-free by soaking in 0.1% DEPC followed by autoclaving (as described above for containers) or by using a commercial RNase decontamination solution according to the manufacturer’s directions. Either method of solution preparation is acceptable. Other options are commercially prepared RNase-free solutions available from several vendors, or recently-introduced alternatives to DEPC treatment.

How Do You Minimize RNA Degradation during Sample Collection and Storage?

RNase is present in all cells and tissues; hence they must be immediately inactivated when the source organism dies. Samples should be immediately frozen in liquid nitrogen, or immediately disrupted in a chaotropic solution (i.e., GITC). In some cases RNase activity can eventually be restored even in the presence of a chaotrope if the extract is not frozen (Amersham Pharmacia Biotech, unpublished observations). In other experiments homogenized tissue has been stored for at least one week at room temperature, or two months at 4°C without any loss of RNA in a lysis buffer (Ambion, Inc., unpublished observations). A commercial RNase inhibitor also exists that can prevent RNA degradation within mammalian tissue, cells, and some plant tissues stored above freezing temperature for long periods. However periodically sampling the integrity of RNA purified from frozen stock materials is recommended in light of reports of RNA degradation in samples frozen under protective conditions.

Mammalian Tissues and Cells

Tissues can be harvested and immediately immersed in liquid nitrogen. However, large pieces of tissue do not freeze instantaneously, allowing RNase to degrade RNA found in the interior of

the sample. The smaller the tissue pieces, the faster it freezes. Once frozen, tissue should be immediately moved to a -70°C freezer, or stored on dry ice until it can be transferred to a freezer for long-term storage. In frozen tissue, RNA may be stable indefinitely, but periodic sampling for RNase degradation is recommended to avoid unpleasant surprises.

If the sample tissue is relatively soft (see the discussion of disruption methods below), and samples are few, they can be harvested directly into the lysis solution, immediately homogenized, and stored up to 12 months at -70°C without affecting RNA quality. Such lysates can be thawed on ice, an aliquot removed for processing, and refrozen. Firm or hard tissue requires more physical disruption as described below.

Mammalian cells are typically easy to homogenize. After a quick wash in culture media to remove debris, pipetting or vortexing in the presence of lysis solution will usually suffice. Cell lysates should be stored at -70°C . Alternatively, washed cell pellets can be quick-frozen by immersing the tube containing the pellet into liquid nitrogen. The tube can then be transferred to -80°C for long-term storage. The disadvantage to freezing cell pellets is that except for very small ones, they will have to be pulverized in liquid nitrogen for RNA isolation.

Bacteria and Yeast

Most gram-negative bacteria can be pelleted and frozen. Small samples (milliliters) of *E. coli* can be lysed and frozen as described above for mammalian cells; larger volumes (liters) will require enzymatic digestion or isolation procedures that incorporate lysis (e.g., an SDS lysis/isolation procedure). Some gram-positive bacteria and most yeast cells resist disruption and require more aggressive methods as described below.

How Do You Minimize RNA Degradation during Sample Disruption?

Fast and complete lysis of any sample is arguably the most critical element of RNA purification. When purifying RNA from a sample type for the first time, test your homogenization procedure for speed, efficiency, and ease of use in a small-scale experiment. A purification procedure involving 20 precious samples is the wrong time to discover the practical limits of an extraction procedure.

RNase inhibition provided by chaotropes and other reagents can be overwhelmed by adding too much starting material. Follow your procedure's recommendation. Scale up if necessary.

Monitor Disruption

Disruption can usually be monitored by close inspection of the lysate. Visible particulates should not be observed, except when disrupting materials containing hard, noncellular components, such as connective tissue or bone. Disruption of microorganisms, such as bacteria and yeast, can be monitored by spectrophotometry. The A_{260} reading should increase sharply as lysis begins and then level off when lysis is complete. Lysis can also be observed as clarification in the suspension or by an increase in viscosity.

Mammalian Tissues and Cells

Most animal tissues can be processed fresh (unfrozen). It is important to keep fresh tissue cold and to process it quickly (within 30 minutes) after dissection. If tissues are necrotic, the RNA can begin degrading *in vivo*. Ideally pre-dispense the lysis solution into the homogenizer, and then add the tissue and begin homogenizing. Samples should never be left sitting in lysis solution undisturbed.

Electronic rotor-stator homogenizers (e.g., Polytron) can effectively disrupt all but very hard or fibrous tissues. In addition, they do the job rapidly. If you have access to an electronic homogenizer, for most tissues, you should use it. If you can only use manual homogenizers, soft tissues can be thoroughly disrupted in a Dounce homogenizer, but firm tissues, however, especially connective tissues, will be homogenized more thoroughly in a ground glass homogenizer or TenBroeck homogenizer (available from Bellco, Vineland, NJ). Very hard tissues such as bone, teeth, and some hard tumors may require a milling device as described for yeast. A comparison of tissue disrupters is described in Johnson (1998). Enzymatic methods may also be used for specific eukaryotic tissues, such as collagenase to break down collagen prior to cell lysis.

Animal tissues and any type of relatively large cell pellets that have been frozen after collection must be disrupted by grinding in liquid nitrogen with a mortar and pestle. During this process it is important that the equipment and tissue remain at temperatures well below 0°C. The tissue should be dry and powdery after grinding. After grinding, thoroughly homogenize the sample in lysis solution using a manual or electronic homogenizer. Processing frozen tissue this way is cumbersome and time-consuming, but very effective.

Mammalian cells are normally easy to disrupt. Cells grown in suspension are collected by centrifugation, washed in cold 1× PBS,

and resuspended in a lysis solution. Lysis is completed by immediate vortexing or vigorous pipetting of the solution. Rinse adherent cells in cold 1× PBS to remove culture medium. Then add lysis solution directly to the plate or flask, and scrape the cells into the solution. Finally, transfer the cells to a tube and vortex or pipette to completely homogenize the sample. Placing the flask or plate on ice while washing and lysing the cells will further protect the RNA from endogenous RNases released during the disruption process.

Plant Tissues

Soft, fresh plant tissues can often be disrupted by homogenization in lysis solution alone. Other plant tissues, like pine needles, can be frozen with liquid nitrogen, then ground dry. Some hard woody plant materials may require freezing and grinding in liquid nitrogen or milling. The diversity of plants and plant tissue make it impossible to give a single recommendation for techniques specific to your tissue. (See Croy, 1993, and Krieg, 1996, for guidance in preparing RNA from plant sources.)

Yeast and Fungi

Lysozyme and zymolase are frequently used with bacteria and yeast to dissolve cell walls, envelopes, coats, capsules, capsids, and other structures not easily sheared by mechanical methods (Ausubel et al., 1995). Sonication, homogenization, or vigorous vortexing in a lysis solution usually follows enzymatic treatment. Yeast can be extremely difficult to disrupt because their cell walls may form capsules or nearly indestructible spores. Bead mills that vigorously agitate a tube containing the sample, lysis buffer, and small beads will completely disrupt even these tough cells within a few minutes. Bead mills are available from Biospec Products, Inc., Bartlesville, OK, and Bio 101, Vista, CA. Alternatively, yeast cell walls can be lysed with hot phenol (Krieg, 1996) or digested with zymolase, glucalase, and/or lyticase to produce spheroplasts, which are readily lysed by vortexing in a lysis solution. Check that the enzyme you select is RNase-free.

To disrupt filamentous fungi, scrape the mycelial mat into a cold mortar, add liquid nitrogen, and grind to a fine powder with a pestle. The powder can then be thoroughly homogenized or sonicated in lysis solution to completely solubilize (Puyesky et al., 1997).

Bacteria

Bacteria, like plants, are extremely diverse; therefore it is difficult to make one recommendation for all bacteria. Bead milling

will lyse most gram-positive and gram-negative bacteria, including mycobacteria (Cheung et al., 1994; Mangan et al., 1997; Kormanec and Farkasovshy, 1994). Briefly, glass beads and lysis solution are added to a bacterial cell pellet, and the mixture is milled for a few minutes. Some gram-negative bacteria can be lysed by sonication in lysis solution, but this approach is sufficient only for small cultures (milliliters), not large ones (liters).

Bacterial cell walls can be digested with lysozyme to form spheroplasts, which are then efficiently lysed with vigorous vortexing or sonication in sucrose/detergent lysis solution (Reddy and Gilman, 1998). Gram-positive bacteria usually require more rigorous digestion (increased incubation time and temperature, etc.) than gram-negative organisms (Krieg, 1996; Bashyam and Tyage, 1994).

Is There a Safe Place to Pause during an RNA Purification Procedure?

Ideally RNA should be purified without interruption, no matter which procedure is used. If a pause is unavoidable, stop when the RNA is precipitated or is in the presence of a chaotrope. For example, when using an organic isolation procedure, the RNA isolation can be stopped when the samples have been homogenized in a chaotrophic lysis solution. They can be stored for a few days at -20°C or -80°C without degradation.

What Are the Options to Quantitate Dilute RNA Solutions?

Spectrophotometry

The most common quantitative approach is to dilute a small volume of the RNA prep to meet the sample volume requirement of the cuvette. If the concentration of your RNA stock is low, the absorbance of the diluted RNA may fall outside the linear range of the spectrophotometer (see Chapter 4, “How to Properly Use and Maintain Laboratory Equipment”).

Cuvettes are commercially available to accommodate sample volumes below $10\mu\text{l}$; some instruments can accept capillaries that hold less than $1\mu\text{l}$. If your spectrophotometer can tolerate these cuvette’s minute sample windows, sample dilution might be unnecessary.

Dilute solutions can be concentrated by precipitation and microfiltration. Centrifugation-based RNase-free concentrators are available from Millipore corporation. (Bedford, MA), and glycogen enhances the precipitation of RNA from dilute solutions (Amersham Pharmacia Biotech, *MRNA Purification Kit Instruction Manual*, 1996). Adjust the NaCl concentration of 1.0ml of an

aqueous solution of RNA to 300mM using a 3 M NaCl stock prepared in 10mM Tris, 1 mM EDTA, pH 7.4. Add 10 μ l of a 10mg/ml glycogen solution (prepared in RNase-free water). Next, add 2.5 ml of ice-cold ethanol. Mix. Chill at -20°C for at least 2 hours, then centrifuge at 4°C for 10 minutes at $12,000 \times g$ to recover the precipitated RNA. Be aware that since it is from a biological source, glycogen can contain protein (e.g., RNase) and nucleic acid (e.g., DNA) contaminants.

The riskiest option is to place your undiluted RNA prep into a cuvette. If this is your only option, carefully rinse the quartz cuvette with concentrated acid (check with your cuvette supplier to determine acid stability) followed by extensive rinsing in RNase-free water. Avoid hydrofluoric acid, which etches quartz and UV grade silica. Concentrated hydrochloric and nitric acid are tolerated by cuvettes of solid quartz or silica, but can damage cuvettes comprised of glued segments. A better option is to treat the cuvette with a commercial RNase decontamination solution.

Fluorometry

An alternative quantitation strategy is staining RNA with dyes such as Ribogreen®, SYBR®Green, and SYBR Gold (all available from Molecular Probes, Eugene, OR). Ribogreen is the most sensitive of these dyes for RNA; it is designed to be detected with a fluorometer for RNA quantitation in solution. With Ribogreen and a fluorometer, 1 to 10ng/ml RNA can be detected. In contrast, both SYBR Green and SYBR Gold are designed to quantify RNA in a gel-based format, and they require the use of a densitometer or other gel documentation system that allows pixel values to be converted into numerical data. This method provides only rough approximations of the RNA loaded on a gel; it is valid for concentrations of 1 to 5 $\mu\text{g}/\text{lane}$. These dyes do not bind irreversibly to the RNA and do not have negative effects on downstream applications.

WHAT ARE THE OPTIONS FOR STORAGE OF PURIFIED RNA?

RNase activity and pH >8 will destroy RNA. For short-term storage of a few weeks or less, store your RNA in RNase-free Tris-EDTA or 1mM EDTA at -20°C in aliquots. For long-term storage, RNA should be stored in aliquots at -80°C in TE, 1mM EDTA, formamide, or as an ethanol/salt precipitation mixture.

TROUBLESHOOTING

A Pellet of Precipitated RNA Is Not Seen at the End of the RNA Purification.

The RNA Pellet Is There, but You Can't See It

- Pellets containing 0.5 to 2.0μg of RNA should be visible but might not be as obvious as DNA pellets of the same mass. RNA pellets can range from clear to milky white in appearance. Pellets typically form near the bottom of the tube, but can also smear along the side depending on the rotor angle. Colored coprecipitants can help to visualize RNA pellets, but use them only if they are RNase-free. Marking the centrifuge tube to indicate the anticipated location of the pellet can help locate barely visible pellets.
- Remove the solution used to precipitate the RNA. This sometimes makes the pellet easier to see.
- Proceed as if a pellet is present, and quantitate the solution via a spectrophotometer, fluorometer, or electrophoresis.

The RNA concentration was too low for precipitation by standard techniques

- The efficiency of RNA precipitation can be increased by adding 50 to 150μg/ml glycogen or 10 to 20μg/ml linear acrylamide to typical salt/ethanol precipitations. Glycogen does not appear to inhibit cDNA synthesis, Northern, or PCR reactions, but it may contain DNA, which could result in confusing RT-PCR results. Linear acrylamide is free of contaminating nucleic acids, but it is neurotoxic. Exercise great caution when handling RNA precipitated with acrylamide. Refer to manufacturers' Material Safety Data Sheets for more information on toxicity of linear acrylamide solutions.

The RNA pellet is truly absent

Sample Source Issues
Was the sample obtained from an unhealthy source? Did the tissue appear to be necrotic? Was the sample quantity insufficient for the purification procedure?
Storage Issues
When originally isolated, was the sample allowed to linger at room temperature, or was it flash frozen immediately?

Was it stored in a frost-free freezer, hence subjected to thawing?
Was the pH of the stored preparation below 8.5?

Homogenization Issues

Was the sample immediately homogenized, or was it left intact for any period of time?
Was the extraction fast, thorough, and complete? Was the RNA too dilute to be effectively precipitated?

Was the Pellet Accidentally Discarded While Removing a Supernatant?

Nonsiliconized tubes decrease the likelihood of this happening.

A Pellet Was Generated, but the Spectrophotometer Reported a Lower Reading Than Expected, or Zero Absorbance

Refer to the troubleshooting example in Chapter 2, “Getting What You Need from a Supplier.”

Did the RNA completely dissolve? Are visible pellet remnants (usually small white flecks) visible?

- Heat the RNA to 42°C, and vortex vigorously. Remove remaining debris by centrifugation. Overdried RNA pellets can be extremely difficult to resuspend; avoid drying with devices like a Speed Vac.

RNA Was Prepared in Large Quantity, but it Failed in a Downstream Reaction: RT PCR is an Example

Is the RNA at fault?

- Did the first strand cDNA synthesis reaction succeed, and the PCR reaction fail?
- Was the quality of the RNA evaluated?
- Was total RNA or poly(A)RNA used in the reaction? Using poly(A)RNA might work where total RNA failed.
- Was the poly(A)RNA purified once or twice on oligo(dT)cellulose. A second round will increase purity but will decrease yield up to 50%.

Is the RT-PCR reaction at fault?

- Did you test the positive control RNA and PCR primers?
- Did you test your gene specific PCR primers?

My Total RNA Appeared as a Smear in an Ethidium Bromide-stained Denaturing Agarose Gel; 18S and 28S RNA Bands Were not Observed

The RNA was degraded

Is it an electrophoresis artifact?

Did the RNA markers produce the correct banding pattern?

If not, the buffers and loading dye could be the problem.

Could the gel be overloaded? 10 to 30 μg /lane of RNA is the maximum amount that should be loaded.

Only a Fraction of the Original RNA Stored at -70°C Remained after Storage for Six Months

The RNA is degraded.

Was the RNA stored as a wet ethanol precipitate or in formamide?

Was the RNA stored as aliquots?

Was the pH of the stored preparation <8.5 ?

Was the RNA frozen immediately after it was isolated?

Did you verify the calculations used to quantitate the RNA?

The RNA adsorbed to the walls of the storage container.

Is the RNA concentration $<0.5 \mu\text{g}/\text{ml}$, which increases the impact of loss due to adsorption?

Is the storage vessel siliconized, which decreases the risk of adsorption?

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